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ScienceDirect
Scientia Horticulturae 115 (2008) 203–208
SCIENTIA HORTICULTURAE
www.elsevier.com/locate/scihorti

Molecular characterization of Kastamonu garlic: An economically important garlic clone in Turkey

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Received 6 February 2007; received in revised form 25 June 2007; accepted 4 September 2007

Abstract

Turkey is one of the major garlic producing country in the World and the significant amount of Turkey's production has been made using a garlic variety called Kastamonu garlic. Therefore, the purpose of this study was to assess genetic relationship of Kastamonu garlic with 20 previously characterized garlic clones collected from different regions of the world using AFLP and locus specific DNA markers. One putative Kastamonu garlic genotype was obtained from Taskopru district of Kastamonu province while another putative Kastamonu garlic genotype was collected from a local farmers' market in Bursa province and called as Kast-Taskopru and Kast-Bursa in this study, respectively. In the UPGMA dendrogram developed by using 120 AFLP markers, Kast-Taskopru was clustered closely over 97% similarity with other non-bolting garlic clones, PI493112, PI493118 and PI383824. This cluster was also supported by bootstrap analysis with 100% bootstrap value. All clones in this cluster also shared same alleles of gene specific DNA markers. However, Kast-Bursa shared 100% polymorphic AFLP markers and gene specific markers with a different garlic clone, PI497951 in another distinct cluster of UPGMA dendrogram and this clustering has also bootstrap value of 100%. These results suggest that Kastamonu garlic is not unique and garlic production in Turkey has been made using several garlic clones, even though most of this production has been sold as Kastamonu garlic due to its high popularity. Therefore, a standard Kastamonu garlic genotype needs to be determined by fingerprinting all available garlic clones cultivated in Kastamonu province and other regions of Turkey.

Keywords: Genetic relationships; AFLP; *Allium sativum* L.; Gene specific markers

1. Introduction

Garlic (*Allium sativum* L.) is classified under *Alliaceae* family (Takhtajan, 1997) and it is widely consumed for its culinary and medical benefits. Currently, garlic has been cultivated world wide although its center of origin is presumed to be Central Asia (Vavilov, 1951; Hong Chong and Etoh, 1996). For centuries, garlic has been clonally propagated, which may be speculated to result in a bottleneck for genetic variation in garlic. However, recent genetic studies revealed the presence of considerable genetic diversity among the garlic clones cultivated around the world or collected from the wild (Pooler and Simon, 1993; Maass and Klaas, 1995; Bradley et al., 1996; Al-Zahim et al., 1997; Ipek et al., 2003; Lampasona et al., 2003; Volk et al., 2004). In diverse garlic germplasm collections, two major morphological differences have been recognized: bolting (hardneck) and non-bolting (softneck) types (Jones and Mann, 1963). Bolting garlic clones have the capacity to produce fully elongated flower stalk and fertile flowers which might have potential for setting true seeds. On the other hand, non-bolting clones rarely produce visible flower stalks. Sometimes, a third group has been described as incomplete bolting types (Takagi, 1990). Incomplete bolting clones show variation among the plants of a clone in terms of bolting characteristic. The flower stalk can be aborted at different stages of elongation, which results in an incomplete flowering phenotype within a clone.

Kastamonu garlic is the most widely grown garlic clone in Turkey due to its high soluble solid content, strong flavor, and long storage capability. A recent study showed that it contains 17.2% crude protein, 0.14% essential oil, and 1779 μcg/kg dimethyl sulphite (Haciseferogullari et al., 2005). Another

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0304-4238/$ – see front matter
doi:10.1016/j.scienta.2007.09.001

important characteristic of Kastamonu garlic is its tightly appressed pseudostem (leaf bases wrapped around the neck of a bulb) and this characteristic makes it an ideal variety for making garlic braids. It has attractive white rose blushed cloves which are surrounded by a white outer skin. This garlic clone rarely grows a visible flower stalk, therefore, it can be considered as a non-bolting garlic type.

Kastamonu garlic is very well adapted to Taskopru district in Kastamonu province which is located in the north western part of Turkey. Since Kastamonu province produces about 17% of Turkey's garlic production mainly using this variety, it is called 'Kastamonu garlic' or 'Taskopru garlic' (SIS, 1998). Kastamonu has a temperate climate in Black Sea costal regions and steppe climate in inland regions, receiving most of its precipitation during spring or winter. Soils are medium calcareous, mostly clay loam in texture, and of slightly alkaline pH in Taskopru district where garlic production is concentrated (Taban et al., 2004). The high quality features of Kastamonu garlic are likely due to the interaction between its genotype and the environment where it has been cultivated.

DNA markers have been extensively used for revealing taxonomic relationship among the plant species, analyzing genetic relationship of plant genotypes and fingerprinting cultivars, since they are not affected from the environmental conditions. Among the molecular marker techniques, amplified fragment lengths polymorphisms (AFLP) which is a PCR-based marker technique (Vos et al., 1995) requires no prior sequence information (Tohme et al., 1996) and can easily generate 100 markers in a single reaction which is useful to discriminate closely related cultivars with a few primer combinations. These properties of AFLP made it an attractive DNA marker system for garlic and, therefore, this technique was successfully applied for genetic characterization and mapping of garlic (Lee et al., 2002; Ipek et al., 2003; Lampasona et al., 2003; Volk et al., 2004; Ipek et al., 2005). In addition, sequence characterization of AFLP markers indicated that sequences of polymorphic AFLP amplicons among garlic clones were highly homologous (Ipek et al., 2006).

Due to the economical importance of Kastamonu garlic for Turkey, the aim of this study was to assess the genetic relationship of Kastamonu garlic with previously characterized garlic clones by Ipek et al. (2003) using AFLP and gene specific markers.

2. Materials and methods

2.1. Plant materials

One Kastamonu garlic clone was obtained from Taskopru district of Kastamonu province after harvest season in 2005 and this is referred to as 'Kast-Taskopru' in this study. This clone had the typical morphological characteristics of Kastamonu garlic described above. Another genotype was obtained from a local farmer market in Bursa province in Turkey since it was also sold as Kastamonu garlic. This garlic clone was named as 'Kast-Bursa' in this study. Some plants from this garlic genotype had incompletely elongated flower stalks which resemble incomplete bolting garlic types. In addition, twenty garlic clones collected from various countries were included to this study to assess their relationships with Kastamonu garlic (Table 1). In our previous study, a large collection of garlic clones were clustered in 10 groups (Ipek et al., 2003) and these 20 garlic clones were selected from that collection to analyze in our present study to represent 10 groups identified in that study. Plant materials were planted to field in November, 2005 and appropriate cultivation practices were applied.

Table 1
Names, origins and flowering data of 22 garlic clones

Clones	Country of origin[a]	Flowering[b]
DDR6807	DE	B
DDR7040	DE	B
DDR7099	DE	B
DDR7116	DE	B
JN/EG	US	B
K/RO	FR	B
Kast-Bursa	TR	IB
Kast-Taskopru	TR	NB
KS/10	FR	B
M/PIT	CZ	B
PI383824	YU	NB
PI493112	PL	NB
PI493116	CZ	B
PI493118	PL	NB
PI497945	PL	B
PI497951	SY	IB
PI515971	US	NB
PI515974	US	NB
Purple	US	B
U074	UZ	B
U079	UZ	B
U094-4	UZ	B

[a] CZ = Czech Republic, DE = Germany, FR = France, PL = Poland, SY = Syria, TR = Turkey, UZ = Uzbekistan, US = United States of America, and YU = Former Yugoslavia.
[b] Flowering data: B = bolting, NB = non-bolting, and IB = incomplete bolting.

2.2. Sampling and AFLP procedures

Young leaves from each clone were lyophilized and DNA was extracted from 100 mg powdered leaf samples in micro centrifuge tubes using a modified CTAB method (Fütterer et al., 1995). DNA concentration of each sample was adjusted to 30 ng/μl for PCR analysis using a TKO 100 Mini-Fluorometer (Hoefer, San Francisco, CA, USA).

AFLP procedure was carried out according to methods described previously by Ipek et al. (2003). EACGG/MCAT, EACGG/MCTC, and EAAGG/MCGA primer combinations were used to generate AFLP markers.

2.3. PCR procedure for locus specific DNA markers

Primer pairs that were previously designed to amplify genomic sequences of garlic genes, alliinase, chitinase, and sucrose 1-fructosyltransferase (SST-1) (Ipek et al., 2005) were used to generate locus specific markers. For this purpose, the

following reaction and thermal cycle conditions were used: each 25 μl PCR reaction contained 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI, USA), with supplied reaction buffer at 1× concentration, 0.8 μM of each primer, dNTPs at 200 μM each, 60 ng template DNA. The reactions were heated to 95 °C for 2 min and exposed to 40 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min, with a final extension step of 5 min at 72 °C. After 40 cycles, reactions were held at 4 °C. Perkin Elmer model 9600 Thermal Cycler was used for amplifications. Amplified products were denatured at 90 °C for 4 min in an equal volume of formamide loading dye buffer and immediately placed onto ice. Denatured PCR products were separated on 6% denaturing polyacrylamide sequencing gels containing 7.5 M urea in 1× Tris-Borate (TBE), 320 μl 10% ammoniumpersulphate (APS) and 30 μl TEMED by running 5–6 μl of reactions at 25 W for 5–6 h. In order to visualize separated fragments, gels were stained using Silver Sequence™ DNA Staining Reagents kit and following the protocol of the manufacturer (Promega).

2.4. Data analysis

All unambiguously scorable polymorphic DNA fragments were manually identified on each autoradiograms for AFLP and on each stained gels for gene specific markers. Polymorphic bands were scored as present (1) or absent (0). A similarity matrix for AFLP markers was prepared according to the coefficient of Jaccard (Sneath and Sokal, 1973) and used to perform cluster analysis using the unweighted pair group method with arithmetic averaging (UPGMA) (Sokal and Michener, 1958) with NTSYS-PC v. 1.80 program (Rohlf, 1993). Dendrogram indicating the estimated similarity among the garlic clones was constructed with the TREE program of NTSYS-PC. Mantel test of significance (Mantel, 1967) was performed to determine the extent of distortion in converting the data into the dendrogram by comparing the original similarity matrix with the cophenetic value matrix calculated from AFLP dendrogram. Bootstrap analysis (Felsenstein, 1985) was also carried out using TREECON 1.3B program (Van de Peer and de Wachter, 1994). In this case, the AFLP data was bootstrapped by resampling 2000 times. During the construction of UPGMA dendrogram, Nei and Li (1979) distance coefficient was used.

3. Results and discussion

AFLP analysis with three primer combinations generated a total of 120 polymorphic markers among the 22 garlic clones. EACGG/MCAT, EACGG/MCTC and EAAGG/MCGA primer combinations generated 44, 43, 33 markers, respectively, averaging 40 polymorphic markers per primer combination.

A Jaccard's similarity matrix was prepared using 120 AFLP markers (Table 2). Similarity among the garlic clones ranged from 0.21 to 1.00. The Kast-Taskopru clone from Taskopru shared highest similarity (0.98) with PI383824 and PI493112 while the Kast-Bursa clone from a local market in Bursa province had identical AFLP markers with PI497951.

Table 2
Jaccard's similarity among 22 garlic clones evaluated in this study using 120 AFLP markers

	KS/10	PI497945	PI497951	PI493112	PI493118	PI383824	Kast-Taskopru	Kast-Bursa	PI515971	PI515974	U074	PI493116	DDR7116	K/RO	DDR6807	U094-4	U079	JN/EG	DDR7099	PURPLE	DDR7040
PI497945	0.97																				
PI497951	0.34	0.30																			
PI493112	0.30	0.23	0.64																		
PI493118	0.25	0.25	0.71	0.97																	
PI383824	0.24	0.22	0.62	0.98	1.00																
Kast-Taskopru	0.32	0.23	0.65	**0.98**	**0.97**	**0.98**															
Kast-Bursa	0.36	0.38	**1.00**	0.58	0.80	0.58	0.58														
PI515971	0.35	0.35	0.66	0.59	0.61	0.57	0.61	0.69													
PI515974	0.32	0.31	0.66	0.64	0.67	0.63	0.65	0.65	0.94												
U074	0.24	0.27	0.28	0.29	0.32	0.32	0.28	0.35	0.30	0.29											
PI493116	0.27	0.28	0.29	0.30	0.31	0.31	0.30	0.34	0.33	0.32	0.95										
DDR7116	0.25	0.25	0.34	0.37	0.38	0.38	0.37	0.43	0.36	0.39	0.67	0.70									
K/RO	0.30	0.33	0.43	0.47	0.51	0.49	0.47	0.54	0.43	0.45	0.49	0.53	0.56								
DDR6807	0.33	0.33	0.39	0.44	0.51	0.49	0.43	0.54	0.38	0.40	0.49	0.51	0.57	1.00							
U094-4	0.32	0.25	0.39	0.37	0.43	0.41	0.38	0.31	0.42	0.43	0.43	0.47	0.40	0.53	0.52						
U079	0.38	0.35	0.42	0.36	0.42	0.38	0.37	0.48	0.46	0.43	0.40	0.42	0.42	0.46	0.46	0.44					
JN/EG	0.37	0.35	0.41	0.34	0.42	0.38	0.36	0.48	0.45	0.42	0.41	0.41	0.40	0.46	0.47	0.43	0.99				
DDR7099	0.37	0.33	0.38	0.34	0.39	0.37	0.36	0.50	0.44	0.42	0.32	0.33	0.30	0.38	0.40	0.37	0.61	0.62			
PURPLE	0.37	0.33	0.38	0.34	0.39	0.37	0.35	0.47	0.43	0.43	0.33	0.34	0.31	0.38	0.41	0.40	0.57	0.57	0.89		
DDR7040	0.29	0.29	0.38	0.32	0.36	0.34	0.32	0.42	0.35	0.38	0.28	0.29	0.30	0.42	0.42	0.37	0.39	0.39	0.44	0.48	
M/PIT	0.31	0.29	0.41	0.35	0.48	0.40	0.37	0.42	0.40	0.40	0.33	0.31	0.34	0.47	0.42	0.41	0.44	0.44	0.46	0.46	1.00

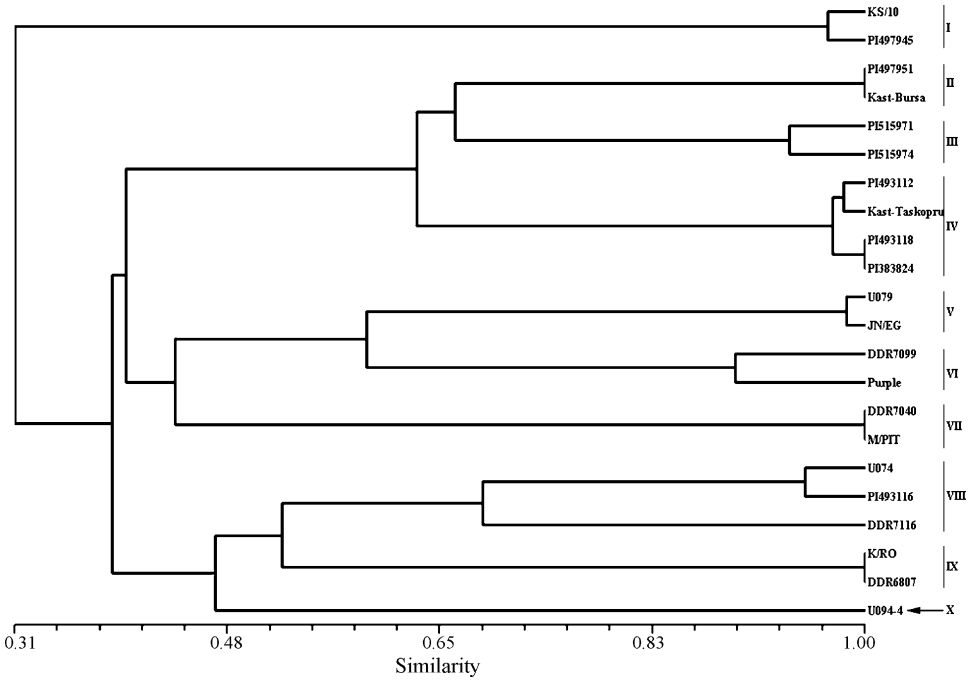

Fig. 1. UPGMA dendrogram constructed using Jaccard's similarity matrix of polymorphic AFLP markers and showing the phenetic relationship among 22 garlic clones analyzed in this study. Roman numerals indicate 10 arbitrary AFLP groups.

Using Jaccard's similarity matrix, a UPGMA dendrogram was constructed to demonstrate the genetic relationship among 22 garlic genotypes used in this study (Fig. 1). To understand how well the data in the Jaccard's similarity matrix (Table 2) was represented by the UPGMA dendrogram (Fig. 1), a matrix of cophenetic values was calculated from the AFLP dendrogram. The comparison of Jaccard's similarity matrix with the matrix of cophenetic values of AFLP dendrogram using the Mantel test (Mantel, 1967) showed that the correlation between these two matrices was very high ($r = 0.95$), suggesting that data in the Jaccard's similarity matrix was represented very well by the UPGMA dendrogram. Bootstrap analysis where data was resampled 2000 times was performed using the TreeCon program to demonstrate how well the tree topology was supported. The nodes having 50% or higher bootstrap values are indicated on the dendrogram (Fig. 2). In the bootstrap tree, 16 of 19 nodes had bootstrap value of 50% or higher and the bootstrap values of 12 nodes were higher than 90%. Fig. 2 shows that the topology of bootstrap dendrogram based on Nei and Li (1979) distance coeffiecient is almost

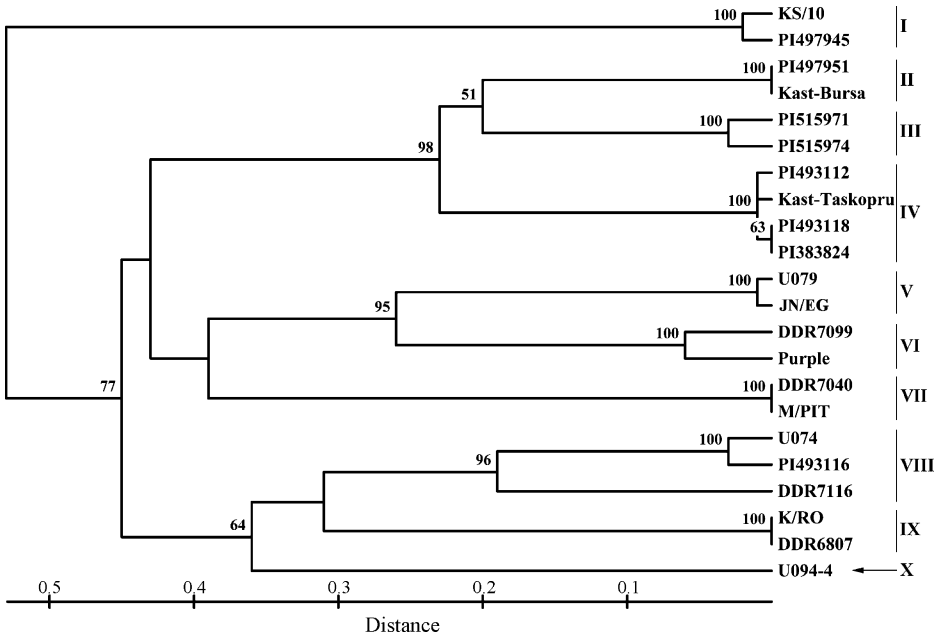

Fig. 2. Bootstrap dendrogram obtained using TreeCon 1.3B, Nei and Li (1979) distance coefficient and UPGMA to show the confidence level of the nodes. Bootstrap values are indicated above the nodes in percentage. Roman numerals indicate 10 arbitrary AFLP groups.

identical to that obtained using Jaccard's similarity coefficient in Fig. 1.

The UPGMA dendrogram based on Jaccard's similarity revealed 10 arbitrary groups among the 22 garlic clones at 67% similarity level (Fig. 1). The similarity between the groups was as low as 30% while the similarity within the group was high except in group VIII (Fig. 1). The two garlic types collected from Turkey did not cluster together in a group but clustered with some other garlic clones collected from various countries with very high similarity. Kast-Taskopru clustered closely with PI493112, PI 493118 and PI383824 garlic clones that were also clustered together in previous study and defined as non-bolting (softneck) types (Ipek et al., 2003), in group IV. However, other putative Kastamonu garlic genotype, Kast-Bursa, shared 100% of its polymorphic AFLP markers with clone PI497951 in group II. PI497951 was identified as an incomplete bolting garlic type in previous study (Ipek et al., 2003). Bootstrap UPGMA dendrogram based on Nei and Li distance coefficient also clustered 22 garlic clones in the same 10 groups at the 0.2 distance level (Fig. 2). These 10 groups were also supported by high bootstrap values (Fig. 2).

Our results demonstrated that Kast-Taskopru, the putative Kastamonu garlic, is closely related to other non-bolting garlic clones, PI493112, PI 493118, and PI383824. While both PI493112 and PI 493118 were collected from Poland, former Yugoslavia was the country of origin of PI383824 (Table 1). On the other hand, PI497951, which shared 100% of AFLP markers with Kast-Bursa, was collected from Syria (Table 1). The clones having a very close genetic relationship with Kast-Taskopru were also morphologically very similar to each other (data not presented). We speculated that these clones might be the same clones. Because of its clonal reproduction habit, garlic clones have likely been exchanged freely among collectors or farmers in different countries world wide. If a clone has good agronomic characteristics, it is likely to be cultivated under different names in different countries. Similarly, Bradley et al. (1996) reported that 'California Early 2' shared 97% of RAPD bands with 'Italian White 1' in their diversity study and they proposed that these two garlic clones might be identical genotypes, although they have different names. In another study with RAPD markers, little RAPD polymorphism (1.1%) was found between the accessions of a garlic relative, *Allium ampeloprasum* var. *babingtonii*, whose population structure appeared to be isoclonal, and this polymorphism was attributed to band instability (Treu et al., 2001). The AFLP system, in contrast to the RAPD system, has been found to be highly reproducible (Vos et al., 1995; Karp et al., 1996). Therefore, we speculate that the few AFLP polymorphisms detected among Kast-Taskopru, PI493112, PI 493118, and PI383824 in AFLP group IV likely resulted from the accumulation of point mutations during the independent clonal reproduction cycles of these clones over many years. Indeed, the AFLP technique can detect single nucleotide polymorphisms between two genotypes (Vos et al., 1995). Overall, our results suggest that Kastamonu garlic is not a unique clone, and that its high quality likely comes from its very good adaptation to the environment in Kastamonu province.

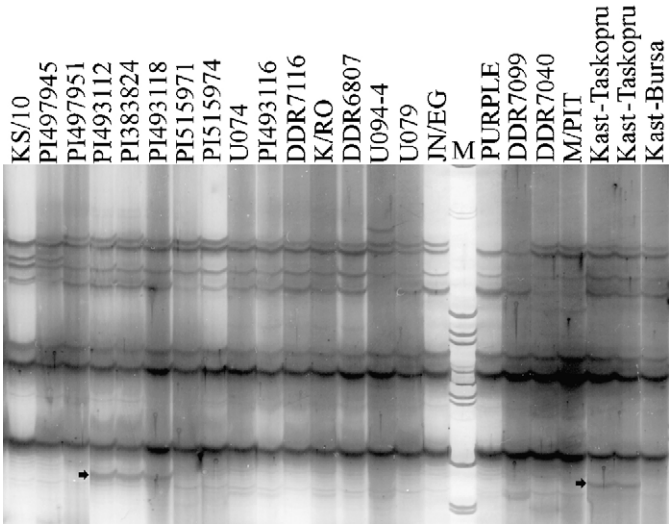

Fig. 3. A polymorphic chitinase allele amplifying only in Kast-Taskopru, PI493112, PI282824, and PI493118 which were tightly clustered in group IV of the UPGMA dendrogram based on AFLP markers (Fig. 1). M is the PBR322-Msp I DNA molecular weight marker. Arrow points to this gene specific marker.

Gene specific primers designed for SST-1, allinase and chitinase generated 23 clear polymorphic alleles from 22 garlic clones. Seven different alleles from SST-1, 11 alleles from allinase and five alleles from chitinase were identified. Garlic clones clustered in an AFLP group (Fig. 1) had the same alleles of all three genes but allelic polymorphisms were observed among the AFLP groups. For example, Kast-Taskopru and the other garlic clones in AFLP group IV had the same alleles of SST-1, allinase, and chitinase (Fig. 3). On the other hand, Kast-Bursa shared the same alleles of these genes with PI497951. One allele of chitinase gene was amplified only in Kast-Taskopru, PI493112, PI 493118, and PI383824 of AFLP group IV (Fig. 3). This might be used as a PCR based gene specific marker to discriminate both Kast-Taskopru and other non-bolting garlic clones in this AFLP group from the other garlic clones cultivated or sold in Turkey.

4. Conclusions

Our results indicated that there was significant genetic variation between Kast-Taskopru and Kast-Bursa. Due to its popularity, it is possible that most of garlic produced in Turkey sold as Kastamonu garlic may include several different garlic genotypes as indicated above. Therefore, the results presented in this study indicate the necessity of collecting a wide range of garlic genotypes currently being used for garlic production in Turkey for DNA fingerprinting. DNA fingerprinting can help to show how much genetic diversity is present among these garlic clones used for garlic production in both the Kastamonu province and in other regions of Turkey. In addition, DNA fingerprinting of the garlic clones used for garlic production in Turkey can allow the identification of a standard Kastamonu garlic clone, and this would assure more uniform garlic production.

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